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TI Purification and characterization of GDP-D-mannose 4,6-dehydratase from porcine thyroid.
AU Broschat K O; Chang S; Serif G
SO EUROPEAN JOURNAL OF BIOCHEMISTRY, (1985 Dec 2) 153 (2) 397-401. Journal code: 0107600. ISSN: 0014-2956.
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Mouse lymphoma cell lines resistant to pea lectin are defective in fucose metabolism.
J Biol Chem. 1980 Oct 25;255(20):9900-6.
PMID: 6159350 [PubMed - indexed for MEDLINE]

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NC 3PO CA13330 (NCI)
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Purification and characterization of GDP-D-mannose 4,6-dehydratase from porcine thyroid

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The enzyme GDP-D-mannose 4,6-dehydratase has been purified 1500-fold from porcine thyroid tissue. The enzyme exhibits a molecular mass of 251 000 Da as determined by sedimentation techniques. Its subunit size was determined as 41 500 Da by dodecyl sulfate gel electrophoresis. The enzyme has a K_m of 3.3 μM with respect to GDP-D-mannose and appears specific with respect to this substrate. The enzyme appears to be inhibited by guanine nucleotides and by guanine nucleotide sugars. It is particularly susceptible to inhibition by GDP-L-fucose. It is suggested that this compound may have a physiological function as an end-product feedback inhibitor.

The *de novo* synthesis of the deoxysugar L-fucose has been observed in bacterial [1–4], plant [5] and mammalian tissues [6, 7]. The first step in the formation of the sugar in all of these systems involves the conversion of GDP-D-mannose to a 4-keto nucleotide sugar intermediate [4, 7]. Although the enzyme concerned with this step, GDP-D-mannose 4,6-dehydratase (EC 4.2.1.47, formerly known as GDP-D-mannose oxidoreductase), has been purified from plants [5] it has not been purified from mammalian tissues. Thyroid tissue is an interesting source of the enzyme since its well-characterized major product, thyroglobulin, contains L-fucose and its synthesis is under effective hormonal control. We wish to report a 1500-fold purification and some properties of the dehydratase from porcine thyroid tissue.

MATERIALS AND METHODS

Materials

The following compounds, reagents and proteins were purchased from Sigma Chemical Company: GDP-D-mannose, GDP-D-glucose, CDP-D-glucose, TDP-D-glucose, UDP-*N*-acetyl-D-glucosamine, UDP-D-galactose, UDP-D-glucose, UDP-D-mannose, L-fucose, blue-dextran, bovine thyroglobulin, rabbit muscle aldolase, yeast hexokinase, ovalbumin, bovine serum albumin, bovine erythrocyte carbonic anhydrase, rabbit muscle, phosphorylase *a*, horseradish peroxidase and yeast alcohol dehydrogenase.

The following materials were purchased from Bio-Rad Laboratories: Bio-Gel HTP, acrylamide, bisacrylamide, Coomassie brilliant blue R 250.

Fluram was obtained from Roche Diagnostics, and GDP-D-[U-^{14}C]mannose from New England Nuclear.

GDP-D-[U-^{14}C]glucose was a gift from Dr George A. Barber, Department of Biochemistry, Ohio State University. GDP-β-L-fucose was a gift from Dr Robert Barker and Dr Paul Rosevear, Department of Biochemistry, Michigan State University.

Blue-dextran–agarose was prepared from Pharmacia brand agarose, Sepharose 4B, and Sigma blue-dextran using the cyanogen bromide method of Ryan and Vestling [15].

Blue-Sepharose was prepared from Pharmacia Sepharose Cl-6B-200 and Sigma reactive blue 2 by the method of Bohme et al. [16].

Purification of GDP-D-mannose 4,6-dehydratase

The following procedure represents a typical isolation from porcine thyroid tissue. All operations were conducted at 4 °C unless otherwise stated.

Preliminary preparation of tissue extracts. Fresh porcine thyroid glands were obtained from the Ohio Packing Company at Columbus and stored on ice in transit from the abattoir. Excess fat and connective tissue were removed and the glands (166 g) homogenized in a Sorvall Omnimixer with 146 ml 0.05 M Tris/HCl buffer ($pH_{25°C}$ 7.5). The crude homogenate was centrifuged at 25000 × *g* for 35 min and the supernatant fluid filtered through glass wool. A second centrifugation was conducted at 75000 × *g* for 3 h. The upper fat layer and lower soft pellet were removed by suction.

Blue-Sepharose step. The supernatant solution obtained from the previous step (172 ml) was mixed with 173 ml blue-Sepharose Cl-6B-200 gel, previously equilibrated in 0.05 M Tris/HCl buffer ($pH_{25°C}$ 7.5), and the slurry was gently swirled every 5 min for a 1-h period. The slurry was then packed into a 173-ml bed-volume column (3.5 × 17.5 cm) and the non-adhering proteins removed with a 1.7-l wash of Tris/HCl buffer. The enzyme was then eluted with 0.5 M KCl. The fractions collected were assayed for enzyme content and those fractions with activity were pooled into a total volume of 735 ml. Aliquots of this pool were used in subsequent steps with the remainder of the pool kept at – 20 °C in 2 M sucrose.

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Abbreviation. SDS, sodium dodecyl sulfate.

Enzymes. GDP-D-mannose 4,6-dehydratase (EC 4.2.1.47); rabbit muscle aldolase (EC 4.1.2.13); yeast hexokinase (EC 2.7.1.1); bovine erythrocyte carbonic anhydrase (EC 4.2.1.1); rabbit muscle phosphorylase *a* (EC 2.4.1.1); horseradish peroxidase (EC 1.11.1.7); yeast alcohol dehydrogenase (EC 1.1.1.1).

A 210-ml aliquot was reduced to 26 ml in an Amicon ultrafiltration unit using a YM-10 membrane. This concentrated solution was subsequently diluted to 173 ml with 0.05 M Tris buffer in order to reduce the KCl concentration to 0.075 M.

DEAE-Sephadex A-50 column step. A 35-ml bed-volume column of DEAE-Sephadex A-50 (2.5 × 7 cm) was prepared and equilibrated overnight with 0.075 M KCl in Tris buffer. The material from the previous step was added to the column at a flow rate of 70 ml/h. The non-binding protein was washed through the column with 350 ml 0.075 M KCl in Tris buffer. GDP-D-mannose 4,6-dehydratase was eluted with 250 ml 0.18 M KCl in Tris buffer. The fractions with enzyme activity were pooled (168 ml) and desalted by passage through a 500-ml bed-volume Sephadex G-25 column (5 × 25.5 cm). The fractions containing protein were combined (176 ml).

Hydroxyapatite I column step. A 12-ml bed-volume (1.5 × 7 cm) hydroxyapatite column was equilibrated with 0.01 M potassium phosphate buffer (pH 7.1). The desalted enzyme solution from the previous step was passed through this column at a flow-rate of 50 ml/h. The non-adhering proteins were removed from the column with a 150-ml wash of 0.01 M potassium phosphate buffer. The adhering protein was eluted with a 0.01 M to 0.15 M linear gradient of potassium phosphate buffer. Fractions containing the enzyme activity were pooled (74 ml).

Combined blue-dextran – Agarose/hydroxyapatite II step. A blue-dextran – Agarose column with a 12-ml bed volume (1.5 × 7 cm) was poured and equilibrated just before use with a solution containing: 5% sucrose, 1 mM EDTA, 0.02% NaN_3, 0.05 M Tris buffer ($pH_{23°C}$ 7.5), and 1 mM ascorbic acid. A 0.6-ml bed-volume hydroxyapatite column was prepared at the same time and equilibrated with 0.01 M potassium phosphate buffer (pH 7.1). The total volume of enzyme solution from the preceding step was diluted with water to a maximum of 30 mM with respect to potassium phosphate and made 5% with respect to sucrose. This sample was passed onto the blue-dextran – Agarose column and washed with 150 ml 0.05 M Tris buffer ($pH_{23°C}$ 7.5). The wash was discarded and the outlet for the blue-dextran column was then connected to the inlet of the hydroxyapatite II column. A 60-ml volume of 200 μM GDP-D-mannose in 1 mM EDTA, 0.02% NaN_3, 5% sucrose, 0.05 M Tris buffer and 1 mM ascorbic acid was then passed into the blue-dextran – Agarose column at the rate of 22 ml/h. Enzyme from the blue-dextran column was thus trapped and concentrated on the hydroxyapatite II column. This latter column was then disconnected, washed with 0.05 M imidazole buffer (pH 7.4) and the enzyme eluted with 10 ml 0.05 M imidazole buffer, containing 2 M sucrose, over a 48-h period at 4°C. Each fraction (0.05 ml) was collected and stored at −20°C immediately following collection.

Molecular mass determination

The native molecular mass of porcine thyroid GDP-D-mannose 4,6-dehydratase was determined by sucrose velocity sedimentation in a 5–20% sucrose gradient using standards of horseradish peroxidase, yeast hexokinase and yeast alcohol dehydrogenase. Subunit molecular mass and the purity of various enzyme preparations were examined through the use of a discontinous sodium dodecyl sulfate (SDS) gel electrophoresis system. Where two-dimensional electrophoresis was employed a modification of the method of O'Farrell was used [9] in that first-dimension tube gels, generated with a continuous phosphate buffer system, were sectioned into 2.0-mm discs; each disc was treated with denaturing buffer and allowed to equilibrate at 37°C for 0.5 h followed by a heat treatment at 100°C for 1 min. Each gel slice was placed in a separate well of previously prepared SDS slab gels together with its denaturing buffer solution. These slabs were then electrophoresed using the discontinuous buffer system of Laemmli and Favre [8] with minor modifications.

Enzyme assay

Assays for dehydratase were conducted according to Liao and Barber [5] with minor modification. The assay volume was 20 μl with final concentrations of 100 μM GDP-D-mannose (containing 9000 dpm GDP-D-[U-^{14}C]mannose), 0.05 M Tris buffer ($pH_{23°C}$ 8.2) and 0.5 mM dithiothreitol. The enzyme sample was added as the last component and the mixture was then briefly stirred with a vortex stirrer and incubated at 37°C for various time intervals. At termination of incubation 2 μmol $NaBH_4$ in 2 μl 0.25 M NaOH were added to reduce the 4-keto intermediate. The reduction was continued for 15 min at room temperature followed by hydrolysis in 0.8 M trichloroacetic acid for 10 min at 100°C to release the individual sugars. The resultant mixture was then spotted on Whatman no. 1 paper chromatography strips (1 × 23 cm) and developed by ascending chromatography in *n*-propanol/ethyl acetate/water (7:1:2) for 2 h at 37°C. The assay strips were marked according to the relative migrations of 6-deoxy-D-talose, D-rhamnose and D-mannose as determined by standards, cut into two segments representing starting sugar (D-mannose) and products (6-deoxy-D-talose and D-rhamnose) and counted by standard liquid scintillation techniques. From a calculation of percentage conversion of D-mannose to products, the amount of product formed (μmol) in the initial reaction volume, per unit time, can be determined.

RESULTS

Enzyme purification

The batch absorption and elution of enzyme using blue-Sepharose as a pseudo-affinity support effectively removes 96% of the extraneous protein at the initial step of purification. Subsequent steps outlined in Table 1 and Fig. 1 lead to an overall purification of the enzyme 1500-fold relative to the specific activity of the crude extract. Individual fractions from the blue-dextran/hydroxyapatite II step were examined for purity on SDS slab gels as described in Materials and Methods. Early fractions from this last elution exhibited a single faint protein band, whose R_F corresponded to a molecular mass of 41 500 Da when compared against appropriate standards. Later fractions from the elution contained the 41 500-Da band plus a second band with an R_F corresponding to a molecular mass of 35 000 Da. Thus the pooled blue-dextran/hydroxyapatite II fraction shown in Table 1 contains a protein impurity which is approximately 50% extraneous protein as judged from an examination of the Coomassie-stained gels. Collection of the early fractions, containing the single SDS electrophoretic component at 41 500 Da, provides a homogeneous enzyme but very low overall yields, i.e. 1.3% recovery of initial enzyme units.

In order to verify that the 41 500-Da subunit is derived from the enzyme, samples from the blue-dextran/hydroxyapatite step were subjected to electrophoresis on non-denaturing tube gels in triplicate. One of these gels was then

Table 1. *Purification table for porcine thyroid GDP-D-mannose 4,6-dehydratase*

Purification step	Total protein	Protein concn	Total activity	Specific activity	Yield	Purification
	mg	mg/ml	nmol/min	nmol min^{-1} mg^{-1}	%	-fold
1. 75000 × *g* supernatant	6000	120	145	0.024	100	1
2. Blue-Sepharose	250	1.19	142	0.57	98	24
3. DEAE-Sephadex A-50	31.9	0.19	121	3.8	83	157
Desalting	29.4	0.18	106	3.6	72	149
4. Hydroxyapatite I	8.5	0.114	93	11.0	63	455
5. Blue dextran/hydroxy-apatite II	0.95	0.237	36	37.6	24	1550

sectioned into 2.0-mm discs and each disc assayed for enzyme activity. A second gel was similarly sliced and the individual discs electrophoretically analyzed on SDS slab gels. A third gel was stained for protein. Fig. 2 depicts the distribution of enzyme activity on the gels. The peak of activity coincided with a faint band of protein seen in the Coomassie-stained gel. The major Coomassie-stained impurity travelled at an R_F of 0.4, well removed from the enzyme activity. Slices from the enzyme peak on the non-denaturing gels were subjected to SDS electrophoresis on a slab gel. A single protein band was detected with an apparent molecular mass of 41 500 Da. It appears from these data that the 41 500-Da band is derived from the enzyme and represents its subunit molecular mass while the faster moving band is derived from a protein impurity.

The apparent oligomer molecular mass was tentatively determined to be 251 000 Da for the enzyme by sucrose velocity sedimentation equilibrium.

Enzyme stability

The stability of the enzyme decreased with increasing purity. In the later purification stages the half-life of the enzyme was of the order of 24 h at 4°C. The enzyme was unstable to freezing at any stage of the purification process except in the presence of very high concentrations of sucrose. Enzyme samples in 2 M sucrose were stored at −20°C with no loss of activity for a period of at least 4 months. The enzyme was not stabilized by sulfhydryl reagents. At low concentrations of dithiothreitol some stimulation of the enzyme was apparent for short periods of time. The more the enzyme was 'aged', prior to treatment with sulfhydryl compounds, the greater was the initial stimulation. Prolonged exposure to sulfhydryl compounds or exposure to high levels of these compounds, however, resulted in an inactivation of the enzyme.

pH optimum

The pH curve for the dehydratase contains a single peak at a pH of 7.1. Greater than 50% of the maximal activity of the enzyme is retained in the range pH 6.5–8.0.

K_m

An apparent K_m of 3.3 μM GDP-D-mannose was obtained as an average from several double-reciprocal plots of substrate concentration versus velocity for the enzyme-catalyzed reaction.

Substrate specificity

The relative unavailability of radiolabelled nucleotide sugars required the examination of such potential substrates by virtue of their ability to compete for the active site on the enzyme. As substrates they should interfere with the enzyme's utilization of GDP-D-[U-^{14}C]mannose. GDP-D-glucose, GDP-L-fucose, CDP-D-glucose, TDP-D-glucose, UDP-*N*-acetylglucosamine, UDP-D-glucose, UDP-D-mannose, UDP-D-galactose and UMP-L-fucose were all examined at a 1 mM concentration in this manner. GDP-L-fucose and GDP-D-glucose are the only nucleotide sugars tested with significant inhibitory effect, i.e. 2% and 35% uninhibited control respectively. As a product of the overall sequence of reactions GDP-L-fucose is probably a feedback inhibitor of the system. GDP-D-glucose, on the other hand, may be an effective substrate. To examine this possibility GDP-D-[U-^{14}C]glucose was substituted for GDP-D-[U-^{14}C]mannose in the assay medium. Subsequent incubation, $NaBH_4$ reduction, hydrolysis and chromatography revealed that the [^{14}C]glucose remained unchanged by the enzyme. Thus GDP-D-glucose is not a substrate for the enzyme. The nucleotides GMP, GDP, GTP, AMP, ADP, ATP, CMP, CDP, CTP, UMP, UDP, UTP and ITP were also examined as potential inhibitors at a 1 mM concentration. Only GDP and GTP showed appreciable inhibitory action, i.e. 27% and 58% of the uninhibited control respectively. Fig. 3 presents the inhibitory effect of GDP-D-glucose, GDP-L-fucose and GDP as a function of inhibitor concentration. GDP-L-fucose is significantly more effective than GDP as an inhibitor of the enzyme.

DISCUSSION

Although the pathway for L-fucose formation was the first 6-deoxy sugar pathway detected [1] the isolation and characterization of the enzymes in this pathway has been largely overlooked, with more emphasis being devoted to the pathway for conversion of D-glucose to L-rhamnose [10]. The single exception has been the purification of the GDP-D-mannose 4,6-dehydratase from plant tissue [5]. That enzyme exhibited a molecular mass of 120000 Da as determined by sedimentation equilibrium studies. The enzyme isolated in this study exhibited a molecular mass of 251000 Da, approximately twice the size of the plant enzyme. This value, taken in conjunction with a subunit molecular mass of 41 500 Da, obtained in this study, might suggest that the native form of the enzyme is a hexamer. Such a conclusion is, of course,

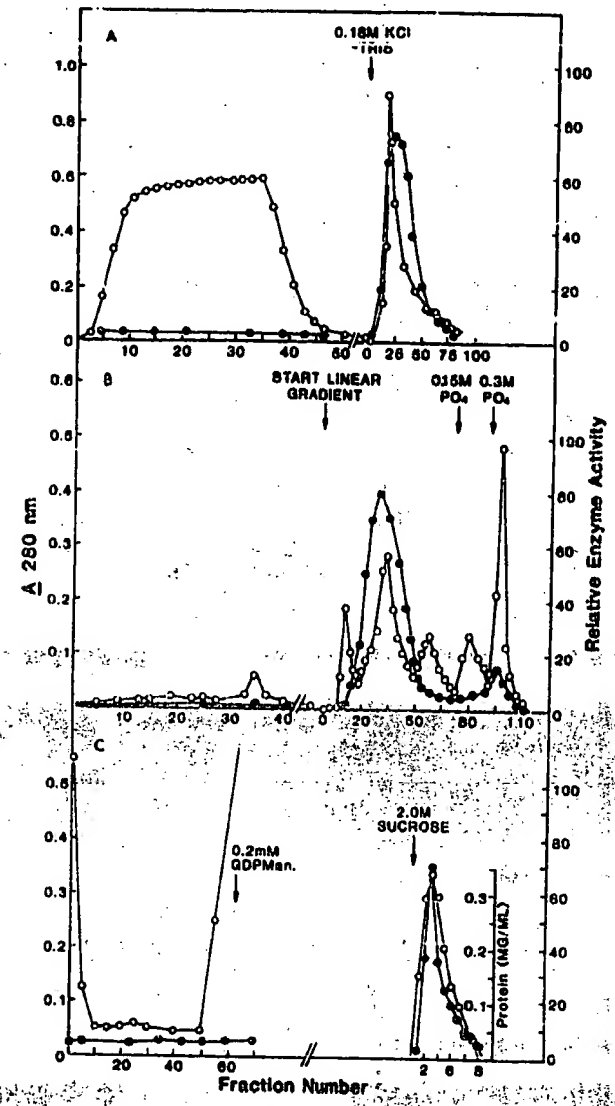

Fig. 1. *Elution patterns for enzyme purification beyond the blue-Sepharose step.* (○) Protein concentration; (●) enzyme activity. (A) Typical DEAE-Sephadex column elution with an initial wash of 0.075 M KCl in Tris buffer (5-ml fractions) and a final elution of 0.18 M KCl in Tris buffer (2-ml fractions). (B) Elution from a typical hydroxyapatite I column with an initial wash of 0.01 M potassium phosphate buffer (pH 7.1, 4.5-ml fractions) followed by a linear gradient of 0.01 – 0.15 M potassium phosphate buffer at the same pH (2-ml fractions) and two step elutions with 0.15 M and 0.3 M buffer. (C) Elution from the blue-dextran and hydroxyapatite II columns. Appropriate fractions from the previous column were pooled, added to the blue-dextran column and washed with 0.05 M Tris buffer (pH 7.5, 3-ml fractions). The blue-dextan column was then connected in tandem to the hydroxyapatite column and enzyme washed into this column with 0.2 mM GDP-D-mannose. The hydroxyapatite column was then separated and the enzyme eluted with 2 M sucrose in 0.05 M imidazole buffer (pH 7.4, 0.5-ml fractions)

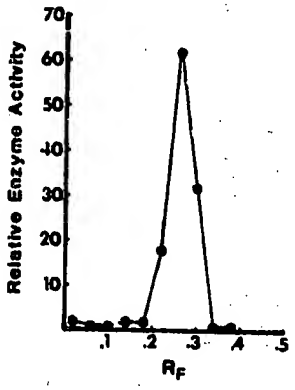

Fig. 2. *Migration of GDP-D-mannose 4,6-dehydratase in 5% polyacrylamide preparative gel electrophoresis.* The gel slices, derived from sectioning the gel into sequential 2.0-mm segments, were each placed in 50 µl buffer overnight and the resulting solutions assayed for enzyme activity on the following morning. The graph points represent gel slices 1 through 10 starting from the origin of the gel

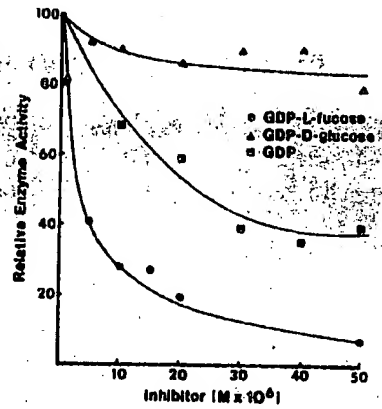

Fig. 3. *The inhibitory effects of various guanine nucleotides on GDP-D-mannose 4,6-dehydratase as a function of inhibitor concentration.* GDP-D-mannose = 5 µM

tentative and requires confirmation by other molecular mass studies using alternative procedures.

Data reported for this enzyme and those obtained from plants support the mechanism whereby a 4-keto sugar intermediate is involved as proposed by Ginsburg for the *Escherichia coli* system and demonstrated to occur for the TDP-D-glucose to TDP-L-rhamnose conversion in *E. coli* [12, 13]. This mechanism would imply a pyridine nucleotide at the active center of GDP-D-mannose 4,6-dehydratase effecting transfer of the C-4 hydrogen to the C-6 position of the deoxy sugar. Although the existence of a pyridine nucleotide in this role has been identified for the TDP-D-glucose to TDP-L-rhamnose conversion [14] no data are available to support this role in the GDP-D-mannose 4,6-dehydratase. If such a nucleotide is present it is tightly bound since it is not removed by extensive dialysis treatment. Nor is the enzyme stimulated by exogenous NAD^+ or $NADP^+$.

GDP-D-mannose 4,6-dehydratase controls the first committed step in the concatenated sequence leading to GDP-L-fucose formation. Consequently it might be expected that end-product inhibition of the enzyme might occur. The highly inhibitory action of GDP-L-fucose, observed in this study, is compatible with such a physiological role for the end-product nucleotide. Similar studies, conducted with bacterial systems, also support this hypothesis [11].

The product of GDP-D-mannose 4,6-dehydratase, GDP-4-keto-D-rhamnose appears to be quite unstable and has never been isolated. This fact has limited study of subsequent enzymes in the pathway for GDP-L-fucose formation. The

availability of GDP-D-mannose 4,6-dehydratase in a high state of purity now permits the generation of quantities of GDP-4-keto-D-rhamnose *in situ* as the substrate for the next enzyme in the L-fucose pathway, presumably an isomerase. We are currently engaged in this study.

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